

**IN VITRO METHOD TO GENERATE  
DENDRITIC LANGERHANS TYPE CELLS**

**Field of Invention**

The present invention relates to a novel *in vitro* method for the generation of dendritic Langerhans type cells from peripheral blood monocytes especially the platelets.

**Background and prior art references**

Dendritic cells (DC), first described by Steinman and Cohn, Z.A. J. Exp. Med. **137**: 1142, 1973 are the most potent antigen presenting cells which initiate immune responses such as the sensitization of T cells restricted by major histocompatibility complex molecules, the rejection of organ transplants and the formation of T cell dependent antibodies. They differentiate from their precursors into so-called "immature" DCs which are present in most tissues (Banchereau, J. and Steinman, R.M. Nature **392**: 245, 1998). The best characterized immature DCs is the Langerhans cells (LC), located above the basal layer of epithelial cells of the skin. Until recently, it was difficult to isolate dendritic cells or Langerhans cells (Steinman, R.M., Lustig, D.S. and Cohn, Z.A. J. Exp. Med. **139**: 1431, 1974). Our knowledge on these important antigen presenting cells has increased dramatically because of success in culturing these cells *in vitro*. Granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) favours the outgrowth of dendritic cells from CD14<sup>+</sup> blood monocytes and CD34<sup>+</sup> cord blood stem cells (Palucka, K.A. Taquet, N. Sanchezchapuis, F. and Gluckman, J.C. J. Immunol. **160**: 4587, 1998). On the other hand, transforming growth factor  $\beta$ 1 (TGF  $\beta$ 1) and IL-4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells (Geissmann, F.C., Prost, J., Monnet, M., Dy, M., Browsse, N. and Hermine, O. J. Exp. Med. **187**: 961, 1998). Culturing purified cord blood CD34<sup>+</sup> cells with GM-CSF and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) also leads to dendritic Langerhans cells (Caux, C., Dezutter-Dambuyant, C., Schmitt, D. and Banchereau, J. Nature **360**: 258, 1992). Several studies have shown that *in vitro* grown dendritic cells or dendritic Langerhans cells can be used in active, specific vaccination schemes to generate protective immunity in several tumour models (Young, J.W. and Inaba, K.. J. Exp. Med. **187**: 7, 1996). (Chakraborty, N.G., Li, L., Sporn, J.R., Kurtzman, S.H., Ergin, M.T. and Mukherji, B. J. Immunol. **162**: 5576, 1991; Hsu, F.J., Benike, C., Fagnoni, F., Liles, T.M., Czerwinski,

D., Taidi, B., Engleman, E.G. and Ltry, R. Nature Medicine **2**: 52, 1996). Controlled studies confirmed the immunogenecity of *in vitro* grown DCs in human, and demonstrated that a single injection of mature DCs rapidly expands T cell immunity (Dhopakar, M.V., Steinman, R.M., Sapp, M., Desai, H., Fossella, C., Krasrsky, J., Donahoe, S.M., Dunbar, P.R., Cerundolo, V., Nixon, D.F. and Bhardwaj, N. J. Clin. Invest. **104**: 173, 1999).

Evidences are being accumulated on the potential of dendritic Langerhans cells in enhancing organ transplant survival. Graft hyporeactivity induced by donor derived dendritic cell progenitors has been reported in animal model (Hirano, A., Fraser, M.O., Jordan, M.L., Takayama, T., Lu, L. and Thomson, A.W. Transplant Proc. **32**: 260, 2000). This is associated with microchimerism and growth of donor dendritic cell progenitors in recipient bone marrow (Khanna, A., Steptae, R.J., Antonysamy, M.A., Li, S. and Thomson, A.W. Transplantation **65**: 479, 1998).

### **Object of the invention**

The main object of the invention is to provide a novel method to generate dendritic Langerhans type cells *in vitro* using platelets.

Another object of the invention is to provide a novel method to generate human dendritic Langerhans type cells *in vitro* from human peripheral blood monocytes using human platelets.

Another object of the invention is to provide a novel method to generate animal dendritic Langerhans type cells from animal bone marrow cells using animal platelets.

Yet another object of the invention is to provide novel method to generate dendritic Langerhans type cells *in vitro* from any mammalian species from their peripheral blood monocytes or bone marrow cells using platelets of the same species or from a phylogenetically close species.

## Summary of the invention

The invention provides an *in vitro* method for developing dendritic Langerhans type cells, for use in immunotherapy against cancer or infectious diseases and enhancement of graft survival in mammals, said method comprising the steps of: culturing cells selected from peripheral blood monocytes and bone marrow cells in a medium containing platelets obtained from the same species or phylogenetically close species and incubating the culture at 30 to 40°C for a period sufficient to enable formation of mature dendritic Langerhans type cells. Using this novel strategy dendritic Langerhans type cells are generated from any mammalian species from their peripheral blood monocytes or bone marrow cells using platelets of the same species or phylogenetically close species. These cells are useful for immunotherapy against cancers or infectious diseases and enhancement of graft survival. Particularly, the present invention involves a novel strategy to generate dendritic Langerhans type cells without the addition of (i.e., omits) exogenous cytokines.

## Detailed description of the invention

The invention provides an *in vitro* method for developing dendritic Langerhans type cells, for use in immunotherapy against cancer or infectious diseases and enhancement of graft survival in mammals, said method comprising the steps of:

- a. culturing cells selected from peripheral blood monocytes and bone marrow cells in a medium containing platelets obtained from the same species or phylogenetically close species; and
- b. incubating the culture at 30 to 40°C for a period sufficient to enable formation of mature dendritic Langerhans type cells.

In an embodiment, the medium comprises RPMI-1640

In yet another embodiment, the cells are cultured for a period of 2 to 8 days.

In still another embodiment, fetal calf serum is added to the medium, with at least 2% being preferable.

In an embodiment, human platelets are added to the medium to develop dendritic Langerhans type cells.

In yet another embodiment, rat platelets are added to the medium containing mice blood cells to develop dendritic Langerhans type cells.

Further, the method to generate human dendritic Langerhans type cells *in vitro* from human peripheral blood monocytes uses human platelets wherein the said method comprises the following steps:

- a. preparing human peripheral blood monocytes;
- b. preparing human platelets;
- c. culturing human peripheral blood monocytes with human platelets for *in vitro* generation of human dendritic Langerhans type cells;
- d. morphological analysis of *in vitro* generated human dendritic Langerhans type cells; and
- e. flow cytometric analysis of *in vitro* generated human dendritic Langerhans type cells.

In another embodiment the method for generation of mouse dendritic Langerhans type cells, comprises the following steps:

- a. preparing mouse bone marrow cells;
- b. culturing of rat platelets;
- c. *in vitro* generation of mouse dendritic Langerhans type cells by culturing mouse bone marrow cells with rat platelets; and
- d. morphological analysis of *in vitro* generated mouse dendritic Langerhans type cells.

One more embodiment, relates to a method to generate dendritic Langerhans type cells from any mammalian species using platelets wherein the said method comprises the following steps:

- a. preparing peripheral blood monocytes and or bone marrow cells;
- b. culturing peripheral blood monocytes or bone marrow cells with platelets of the same species or phylogenetically close species for *in vitro* generation of dendritic Langerhans type cells;
- c. morphological analysis of the *in vitro* generated dendritic Langerhans type cells; and

- d. flow cytometric analysis of the *in vitro* generated dendritic Langerhans type cells.

**Brief description of the accompanying drawings**

**Fig.1.** Comparison of morphology of human dendritic Langerhans type cells grown *in vitro* from human peripheral blood monocytes using human platelets or recombinant cytokines. Ten days cultures are shown. Magnification,  $\times 300$ .

- A. Medium RPMI only (without FCS);
- B. Medium RPMI without FCS+ Platelets;
- C. Medium RPMI containing 2% FCS + rhGMCSF + rhIL-4
- D. Medium RPMI containing 2% FCS + Platelets

**Fig. 2.** Immunophenotyping of human dendritic Langerhans type cells generated *in vitro* from human peripheral blood monocytes using human platelets. One colour dot-plot is shown. The percent positive cells are given in the upper left or lower right quadrants.

**Fig.3.** Mouse dendritic Langerhans type cells generated *in vitro* from mouse bone marrow using rat platelets. BALB/c mice bone marrow cells ( $300 \times 10^3/\text{well}$ ) were cultured in RPMI-1640 containing 10% FCS with or without rat (Sprague-Dowley) platelets ( $2 \times 10^8/\text{ml}$ ) in 24 well tissue culture plates. Phase contrast micrographs of five days cultures are shown. Magnification,  $\times 300$ .

- A. Medium RPMI containing 10% FCS; and
- B. Medium RPMI containing 10% FCS + rat platelets

The invention is illustrated by the following examples which should not be construed as limitations on the inventive scope embodied herein.

**Example 1**

**Preparation of human blood monocytes**

Heparinised (10 U/ml) normal human whole blood was subjected to Ficoll/Hypaque density gradient centrifugation to get peripheral blood mononuclear cells (PBMC). PBMC were washed ( $\times 5$ ) in RPMI-1640 medium without fetal calf serum (FCS). PBMC

were then allowed to adhere in 24 Well tissue culture plates ( $1.5 \times 10^6$  cells/well) for three hours. Non-adherent cells were removed by gentle swirling followed by washing ( $\times 3$ ). Adherent monocytes were cultured at 37°C in 5% CO<sub>2</sub> with or without human platelets. Recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF) and recombinant human interleukin-4 (rhIL-4) were added in parallel cultures for comparison purpose.

**Example 2:**

**Preparation of human platelets**

Heparinised human whole blood was centrifuged for 15 mins at 2000 rpm to pellet down the RBC, mononuclear cells (PBMC) and Polymorphonuclear neutrophils (PMN). The plasma containing the platelets was collected and the centrifugation was repeated to remove any residual RBC, PBMC and PMN. The plasma was then subjected to high speed centrifugation (10,000 rpm for 10 min) to pellet down the platelets. The platelets were washed ( $\times 2$  with RPMI-1640 without FCS) and counted.

**Example 3:**

**In vitro generation of human dendritic Langerhans type cells by incubating human blood monocytes with human platelets.**

Adherent monocytes were cultured in RPMI-1640 (without FCS) in the presence or absence of human platelets ( $2 \times 10^8$ /ml of culture) at 37°C in 5% CO<sub>2</sub>. In selected experiments, RPMI-1640 containing 2% FCS was also used instead of RPMI-1640 without FCS. No medium change was done. Cells were kept in the same wells for up to 60 days with >95% viability.

**Example 4:****Generation of mouse dendritic Langerhans type cells *in vitro* from mouse bone marrow using rat platelets**

BALB/c mice bone marrow cells ( $30 \times 10^5$  cells/well) were cultured in RPMI-1640 medium containing 10% FCS in 24 well plates. Rat platelets were prepared from rat plasma and added ( $2 \times 10^8$  cells/ml) to mouse bone marrow cultures. Cultures were incubated at 37°C in 5% CO<sub>2</sub>. Rat platelets were prepared from the whole heparinised blood of Sprague-Dowley rats using the same method as for human platelets.

**Example 5:****Morphological analysis**

*In vitro* generated human and mouse dendritic Langerhans type cells were analysed under phase contrast microscope.

**Example 6:****Flow cytometry**

Immunophenotyping of *in vitro* generated human dendritic Langerhans type cells was performed by flow cytometry using FACS Calibur (Becton Dickinson, USA) flow cytometer, and the following human cell surface marker specific monoclonal antibodies (mAb): anti-CD3, anti-HLADR, anti-CD19, anti-CD40, anti-CD1a, anti-CD1b, anti-CD80, anti-CD83 and anti-CD86 (purchased from Pharmingen, USA).

**Results****Human platelets induce generation of dendritic Langerhans type cells *in vitro* from human peripheral monocytes**

When human peripheral blood monocytes were cultured in RPMI-1640 medium without FCS, very few cells differentiated to strongly adherent macrophages and most of the cells died (Fig. 1A). When human monocytes were cultured in RPMI-1640 without FCS in the presence of rhGM-CSF and rhIL-4, there was hardly any transformation to immature DC with typical dendritic processes (not shown). However, when human

peripheral blood monocytes were cultured in RPMI-1640 containing 2% FCS in the presence of 500 U/ml of rhGM-CSF and 500 U/ml of rhIL-4, transformation to immature dendritic cells (DC) with typical dendritic processes was noticed, as expected (Fig.1C). In parallel cultures of human monocytes in FCS free medium when autologous platelets were added instead of rhGM-CSF and rhIL-4 proliferating cells with dendritic processes started appearing within five to six days. Fig.1B shows growing colonies that developed when autologous platelets were added to serum-free human monocyte cultures. Morphologically these cells were similar to that generated in the presence of rhGM-CSF and rhIL-4. Of course, the presence of 2% FCS in human monocyte cultures containing autologous platelets accelerated the process of colony formation of cells with typical dendritic morphology. Typical dendritic processes of these cells grown in RPMI-1640 containing 2% FCS and autologous platelets are shown in Fig.1D. Platelets collected from allogeneic donors were as effective as autologous platelets in inducing growth of cells with dendritic processes from human monocytes.

Immunophenotyping of these cells ruled out the possibility of contaminating T or B cells as CD3<sup>+</sup> and CD19<sup>+</sup> cells in these cultures were 0.2% and 1.35% respectively (Fig.2). The cells showed strong reactivity with anti-HLA-DR, anti-CD40 and anti-CD86 mAbs. The reactivity with anti-HLA-DR, anti-CD40 and anti-CD86 were 98.4%, 71.5% and 57.3% respectively. However, only approximately 20% of the cells expressed CD1a, CD1b, CD80 or CD83 (Fig.2). Taken together, these data suggest that the growing cells with typical dendritic morphology that were generated when human monocytes were cultured in the presence of autologous platelets are dendritic Langerhans type cells.

#### **Rat platelets induce generation of mouse dendritic Langerhans type cells *in vitro* from mouse bone marrow**

To extend our finding in animal models, BALB/c mice bone marrow cells were cultured in RPMI-1640 medium containing 10% FCS and 50 µM β-mercaptoethanol in the presence or absence of human platelets. Human platelets did not induce growth of cells with dendritic morphology from mouse bone marrow (not shown). However, when mouse bone marrow cells were cultured in the same medium in the presence of recombinant mouse granulocyte macrophage colony stimulating factor (rmGM-CSF),

cells with dendritic morphology started growing, as expected (not shown). Interestingly, when platelets collected from Sprague-Dowley rat plasma were added to mouse bone marrow cell cultures in RPMI-1640 containing 10% FCS and 50 µM β-mercaptoethanol, growing colonies with dendritic morphology started appearing from five days of incubation (Fig. 3B). When mouse bone marrow cells were cultured in the absence of rmGM-CSF or rat platelets no such colonies with dendritic morphology were seen, only some adherent macrophages were visible (Fig. 3A). Our data indicate that platelets from autologous or allogeneic normal human donors can generate dendritic Langerhans type cells from human peripheral blood monocytes *in vitro*. Although human platelets were ineffective in generating dendritic Langerhans type cells from mouse bone marrow, platelets from rats could induce the same. This is possibly because rat is phylogenetically more close to mouse than human. Thus, dendritic Langerhans type cells could be generated from any mammalian species from their peripheral blood monocytes or bone marrow cells using platelets of the same species or phylogenetically close species without the addition of any exogenous cytokines.

### **Application**

*In vitro* generated dendritic Langerhans type cells from human peripheral blood monocytes using human platelets may have potential application in immunotherapy against human cancers or infectious diseases. Another potential application is in enhancement of organ transplant survival by inducing donor graft-specific tolerance. *In vitro* generated dendritic Langerhans type cells from animals may be useful in developing immunotherapeutic strategies for cancer and infectious diseases in animal models. These cells may also prove useful in studying tolerance in animal models.

### **Advantage**

The conventional method of generating dendritic Langerhans type cells *in vitro* requires addition of recombinant cytokines like GM-CSF, IL-4 and TGF-β1 to peripheral blood monocyte or bone marrow cultures. These cytokines are expensive. Therefore, generation of dendritic Langerhans cells *in vitro* using the conventional method is costly. On the

other hand, our novel method of generating dendritic Langerhans type cells does not require the addition of any exogenous cytokines to monocyte or bone marrow cultures. Only platelets collected from the same species or from a phylogenetically close species can replace the requirement of exogenous cytokines. Platelets are easy to get and are inexpensive to prepare. Using this novel method large numer of dendritic Langerhans type cells are generated *in vitro* very easily and inexpensively from any mammalian species including human.

The human dendritic Langerhans type cells generated *in vitro* following the method of the invention is useful for immunotherapy against human cancers and infectious diseases. The human dendritic Langerhans type cells generated *in vitro* is effective in enhancing survival of organ transplants in human. *In vitro* generated dendritic Langerhans type cells from animals may be useful in developing immunotherapeutic strategies for cancer and infectious diseases in animal models. *In vitro* generated dendritic Langerhans type cells from animals may be useful in studying tolerance in animal models.